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TITLE: Targeting Midbodies in Ovarian Cancer Stem Cells as a Therapeutic Strategy

PRINCIPAL INVESTIGATOR: Stephen Doxsey

CONTRACTING ORIGANIZATION: Univeristy of Massachusetts, Worcester, MA 01655

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Fort Detrick, Maryland 21702-5012

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E-Mail: Stephen.Doxsey@umassmed.edu

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University of Massachusetts
373 Plantation Street
Worcester, MA 01605

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The etiology, development and progression of ovarian tumorigenesis are among the least understood of all human malignancies. A major difficulty in the treatment of ovarian cancer is tumor recurrence and chemoresistance. One explanation for these impediments to a cure is that a subset of cancer cells, 'cancer stem cells', is both the source of ovarian cancer and a major contributor to the refractory nature of ovarian cancer to chemotherapeutic challenge. A better understanding of ovarian cancer stem cells and their contribution to ovarian cancer is essential and may be the best strategy to ameliorate the disease. The cancer stem cell theory has been embraced as an attractive hypothesis for generating and propagating human tumors. Putative ovarian cancer stem cells have been identified in human and used to induce tumors and serial tumor propagation in mice. However, there has been limited success in therapeutic targeting of CSCs for ovarian tumor eradication.

15. SUBJECT TERMS
Ovarian cancer, stem cells, midbody, mitosis, microtubules

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Unclassified	Unclassified	Unclassified	Unclassified	41	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION: Ovarian cancer is a disease of uncontrolled cell division. Cell division normally creates two genetically identical daughter cells through severing of a cytoplasmic bridge that interconnects them. The midbody is an organelle within the bridge that is involved in severing. Previously, midbodies (MBs) were thought to be lost from cells after division, but we showed that, in cancer cells, they can be retained, accumulated and increased with tumor grade. In this proposal, we identified putative MB-containing ovarian cancer cells and targeted them for chemotherapeutic elimination of ovarian cancer-related phenotypes. We tested if MBs were present in putative ovarian cancer stem cells from multiple ovarian cancer cell lines, if MB-containing ovarian cancer cells had increased tumorigenic potential and if MB degradation decreased tumor potential (Task 3).

2. KEYWORDS: MB, midbody; CSCs, cancer stem cells, NBR1, neighbor of BRCA1, NipSnip2, binding partner of BRCA1, BRCA1, breast cancer 1 gene. MT, microtubules.

3. OVERALL PROJECT SUMMARY: Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes

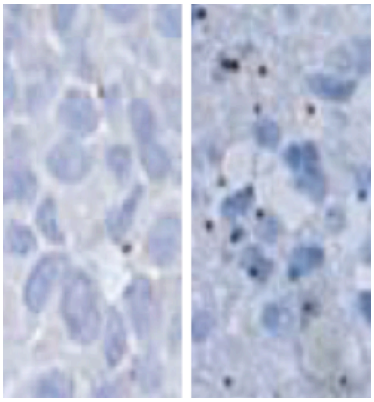

Fig. 1. MBs (brown) in normal (left) and ovarian cancer tissue (right).

Task 1. Test whether MBs are present in ovarian tumors (Fig. 1) and in ovarian CSCs in vitro.

We isolated ovarian CSCs from cell lines and tested them for enrichment of MBs. As in our previous work with other cancer cells (Kuo et al, 2011), we successfully isolated the side population of SKOV3 cells (putative cancer stem cells) based on their ability to use the ABC transporter to pump out the DNA dye Hoechst 33342 (the so called side population). We also isolated putative CSCs by flow cytometry of the fluorescent-tagged cell surface CSC markers (CD133+, CD44+, CD117+) (Dyall et al., 2010; Ponnusamy et al., 2008).

In both strategies, these putative CSCs were fixed, spun onto coverslips and stained for MBs using two different and effective MB markers, MKLP1 and MgcRacGAP. There was near complete (98.8+/- 0.3%) concordance in MB staining using these two different markers. In both CSC preparations MBs were found to be significantly higher in the CSC fraction (77+/- 11.1%

and 92+/-4.3%) versus the nonCSC populations (7.1% and 6.4%). (n=4 experiments/isolation procedure).

During these analyses, we unexpectedly discovered that a large number of canonical ovarian oncogenes and tumor suppressors localized to MBs in epithelial ovarian cancer cells. These included HER-2/neu, c-myc and K-ras, p53, BRCA1, BRCA2 and others. This result provided a potential molecular mechanism for the tumorigenic property of MBs and could serve as a novel therapeutic method for treating ovarian cancer in future studies. To our knowledge, this is the first report on ovarian cancer proteins on midbodies. We hope to garner additional funding to pursue this new discovery and determine how MB binding of these proteins influences ovarian tumor cell.

Task 2. Test the tumorigenic potential of MBs.
We next tested MB-positive cells that were isolated based on MB fluorescence. We used the robust MB marker, MKLP-1 to construct an MKLP-1-GFP fusion protein in both SKOV3 and OVCAR-3 cells. We used flow cytometry to isolate MB-positive cells from these populations. To assay for tumor potential we grew cells in soft agar (anchorage-independent growth). We observed a dramatic increase in soft agar growth in MB-positive SKOV cells, colony #: 107+/-I 6, versus MB-negative SKOV cells, 25.2+/-0.2, $p<0.001$ and a lower but significant increase in MB-positive OVCA cells (OVCAR-3), colony #: 79+/-3.1 vs. 11+/- 1.1. We did not address why there were differences in the degree of soft agar growth between these cells lines, but rather, we focused on the trend, namely that MB-positive cells vs. MB-negative cells have greater tumor-like potential.

We used an independent method to test for the ability of MBs to enhance tumor potential. In this assay, we prevented receptor-mediated degradation of MBs (figure right) by depleting the MB-autophagy-receptor, NBR1. shRNA depletion of NBR1 as in our previous studies (Kuo et al, 2011) blocked autophagic degradation of MBs and increased the percent of MB+ cells by a level similar to those in Kuo et al., 2011 and this was accompanied by an increase in soft agar growth by percentages similar to those observed for cells isolated by MKLP-GPF, above (4.13-fold greater than controls). During the course of this work, we discovered another autophagy protein, NipSnip2. It had an enhanced effect on MB degradation when depleted compared to NBR1 (8.19-fold increase in colony number over control (scrambled shRNA).

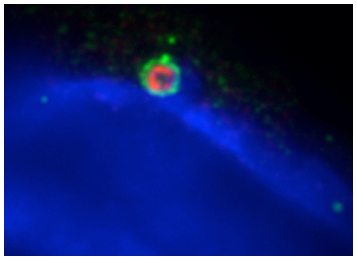
Fig. 2. MB in autophagosome OVCAR3 cell, blue, nucleus, red, MB, green, autophagic vacuole

Task 3. ***Test if specific targeting of MBs for autophagic degradation is a therapeutic strategy for ovarian cancer.***
We showed that expression of NBR1-GFP or NipSnip2-GFP increased autophagy-mediated degradation of MBs over GFP control cells (~ 4-fold fewer MBs than GFP control, Fig. 3) in two different ovarian cancer cells (SKOV3, OVCAR-3) presumably through more efficient binding of these proteins to MBs facilitating their uptake into the MB-selective autophagy pathway. GFP and the GFP-tagged proteins were all expressed at the same levels then analyzed

for soft agar colony number. Soft agar colony number was decreased in GFP-NBR1 expressing cells vs. GFP alone expressing cells with fold differences very similar to that observed in other cancers in our studies 74+/-2.1 vs. 11+/-0.7; Kuo et al., 2011). This result has important implications for ovarian tumor therapy.

From all of these studies we conclude that MBs influence the tumorigenic-like properties in ovarian cancer cells. More specifically, increasing MBs in ovarian cancer cells increases tumor-like activity whereas decreasing MBs decreases tumor-like properties of cells. Future studies will be designed to test if the MB-bound oncogenes and tumor suppressors (above) play a role in this process. We completed nearly all the proposed work on in vitro cell biology studies. This work took more time than anticipated, so we were unable to make progress on the mouse orthotopic tumor experiments. We hope to address this task in the near future.

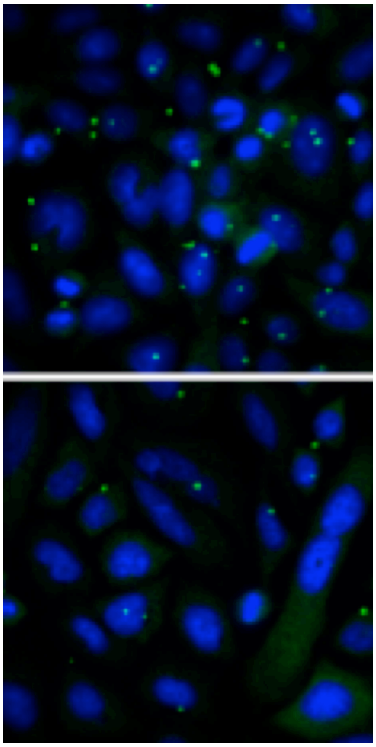

Fig. 3. MBs in OVCAR3 cells expressing GFP (upper) or NBR1-GFP (lower).

4. KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field. For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

*Midbodies are inherited by one daughter cell and accumulate in ovarian cancer cells.

*Midbodies are present in ovarian cancer stem cells and their presence is associated with increased in vitro tumor potential over MB-negative ovarian cancer cells.

*Midbodies serve as scaffolds for anchoring cancer oncogenes, tumor suppressors and cancer stem cell proteins.

*MB-bound cancer oncogenes and tumor suppressors could serve as novel targets for ovarian cancer therapies.

*NBR1 expression effectively eliminates ovarian cancer cells. Expression of the NBR1 interacting protein, NipSnip2, also decreases ovarian cancer cell growth in soft agar. These approaches could become ovarian cancer therapies.

*MB-positive ovarian cancer cells contain stem cell antigens.

5. CONCLUSION: Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions,

innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The results reported in this funding period show promise toward ovarian cancer therapies. These approaches could ameliorate ovarian tumors in military personnel and could be used by medical personnel. Several lines of investigation suggest this could be accomplished: MBs enhance ovarian cancer whereas decreasing MBs kills ovarian cancer cells. Overexpression of autophagy proteins (NBR1, NipSnip2) decreases MBs and kills ovarian cancer cells. This is tumor cell specific because normal cells do not accumulate MBs. MB-positive ovarian cancer cells appear to be ovarian cancer stem cells. This suggests that MB targeting for ovarian cancer therapy will target the cell that is the most insidious of all ovarian cancer cells, the ovarian cancer stem cell. In turn, MB+ ovarian cancer stem cells are likely to be the cells that are drug resistant, recurrent and form metastatic lesions, all of which have been the most difficult to treat. The finding that MBs anchor ovarian cancer oncogenes, tumor suppressors and cancer stem cell proteins suggests that they may serve as scaffolds for ovarian tumor pathways. We expect the work will ultimately have a sustained and significant impact on ovarian cancer for a number of additional reasons: We provide a new understanding of ovarian cancer etiology, namely the finding that an organelle never before associated with cancer may in fact be a key player in ovarian tumor development and progression. We also believe that we have identified a new and effective ovarian cancer stem cell identification strategy based on a novel and atypical biomarker, the MB. We believe that our laboratory is the only one in the world working in this area of cancer biology. We will follow up this work by testing for the significance of cancer-associated proteins that are bound to MBs. We will attempt to mislocalize these proteins from MBs and ask if there are changes in tumor potential. This is a novel and interesting result that makes sense in terms of the mechanism by which MBs confer tumor like properties to cancer cells.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press: None

(2) Peer-Reviewed Scientific Journals:

Kuo et al., Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. Nature Cell Biology 13: 1467, 2011. PMID: 21909099 PMCID: PMC4208311 *(See highLights in Current Biology 21, R958-59; Nature ReviewsMolecular Cell Biology 12, 690-1, 2010; "The Scientist" September 11, 2011; The Keck Foundation Annual Report 2011, p. 8-10)*

(3) Invited Articles:

Chen C-T, Ettinger A, Huttner, W, and Doxsey S. Resurrecting Remnants: The lives of Post-Mitotic Midbodies. Trends Cell Biol. 23, 118-28, 2013. PMID: 23245592. PMCID: 4196272

Kuo TC, Doxsey S. Fates and roles of post-mitotic midbodies beyond cytokinesis. Cell Cycle. 11, 7-8, 2012. PMID: 22157094

Chen C-T, Hehnly H, Doxsey S, Orchestrating vesicle transport, ESCRTs and kinase surveillance during abscission. Nature Reviews Molecular Cell Biology, 13:483-8, 2012. PMID: 22781903. PMCID: PMC4215936

(4) Abstracts:

1. T-C. Kuo1, C-T. Chen1, D. Baron1, T. T. Onder2, S. Loewer2, C. M. Weismann1, G. Q. Daley2,3, S. Doxsey1. Midbodies contribute to cellular reprogramming and tumorigenicity by evading autophagy. American Society of Cell Biology 2011. 1Molecular Medicine, UMass Medical School, Worcester, MA, 2 Stem Cell program, Children's Hospital Boston, Boston, MA, 3Howard Hughes Medical Institute, Chevy Chase, MD.

2. T. Kuo, C. Chen, D. Baron, S. Doxsey. Midbodies contribute to cellular reprogramming and tumorigenicity by evading autophagy. American Society of Cell Biology, 2012. UMass Medical School, Worcester, MA

3. J. Schiel, C-T. Chen, S.J. Doxsey. The mature mother centrosome influences abscission site specification. American Society of Cell Biology 2013. UMass Medical School, Worcester, MA.

4. B.A. Cortez 1. P.R. Teixeira 2, S. Redick 3, S. Doxsey 3, G. Machado-Santelli. 1Understanding how aneuploidy and multipolar mitosis are formed after chrysotile and vincristine treatments. American Society of Cell Biology 2013. 1Depto Genetica e Biologia Evolutiva, University of São Paulo, São Paulo, Brazil, 2Department of Cell and Developmental Biology, University of Sao Paulo, Sao Paulo, Brazil, 3Univ Massachusetts Med Sch, Worcesteer, MA.

a. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

2014 (scheduled or attended)**:**

1. Massachusetts General Hospital, MGH Department of Medicine (scheduled)
Host: Dr. Roy Soberman

2. World Congress on Cell Research: Cancer Stem cells, Chicago, USA (scheduled)
Host: Jessi Casper

3. UMass Medical School, Program in Molecular Medicine, Retreat (attended)

7. INVENTIONS, PATENTS AND LICENSES: List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

None

8. REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

Manuscripts. We published papers on many of the findings discussed in this report.

1. Kuo et al., Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. Nature Cell Biology 13: 1467, 2011. PMID: 21909099 PMCID: 4208311 *(See highights in Current Biology 21, R958-59; Nature Reviews Molecular Cell Biology 12,690-1, 2010; "The Scientist" September 11, 2001; The Keck Foundation Annual Report 2011, p. 8-10).*

2. Kuo TC, Doxsey S. Fates and roles of post-mitotic midbodies beyond cytokinesis. Cell Cycle. 11, 7-8, 2012. PMID: 22157094

3. Chen C-T, Ettinger A, Huttner, W, and Doxsey S. Resurrecting Remnants: The lives of Post-Mitotic Midbodies. Trends Cell Biol. 23, 118-28, 2013. PMCID: 4196272

4. Chen C-T, Hehnly H, Doxsey S, Orchestrating vesicle transport, ESCRTs and kinase surveillance during abscission. Nature Reviews Molecular Cell Biology, 13:483-8, 2012. PMID: 22781903 PMCID: 4215936

Cell lines.
We established SKOV3 cell lines expressing GFP-MKLP1, which targets to midbodies.
We established SKOV3 cell lines expressing GFP-Cep55, which targets to midbodies.
We established SKOV3 cell lines expressing GFP-NBR1, which targets to autophagosomes.
We established SKOV3 cell lines expressing GFP-NipSnip2, which targets to autophagosomes
We established OVCAR-3 cell lines expressing GFP-MKLP1, which targets to midbodies.

Funding opportunities.

A postdoctoral fellow in the laboratory, Dr. Heidi Hehnly, received an NIH K999 award based, in part, on the work in this proposal.

Invited seminars based on work in this project (P.I. and/or lab members)

2011:

03/2011	Frontiers in Science, UMass Medical School, Worcester MA
05/2011	Polycystic Kidney Disease Conf., Harvard Medical School, Boston
08/2011	Biology of Aging, Ellison Foundation Meeting, Woods Hole, MA
06/2011	**Plenary Lecture**, Molecular Medicine, Istanbul, Turkey
08/2011	**Plenary Lecture**, Molecular/Cell Biology, Beijing, China
09/2011	EMBO Workshop, Chromosome Function, Cape Sonio, Greece
10/2011	Centrosomes and Spindle Pole Bodies, Barcelona, Spain
11/2011	American Society of Nephrology, Philadelphia PA
12/2011	American Society of Cell Biology, Denver CO

2012:

04/2012	University of Colorado at Denver, Denver CO
05/2012	**Plenary Lecture**, Congress, Cell Biology, Rio de Janeiro, Brazil
05/2012	**Plenary Lecture**, Cilia Structure/Function, Hanko Isle, Norway
05/2012	Cilia in Development and Disease Conference, London England
06/2012	**Plenary Lecture**, Stockholm, Sweden
06/2012	University of Pennsylvania, Philadelphia, PA
06/2012	National University of Ireland, Chromosome Biology, Galway
11/2012	International Drug Discovery Science/Technology, Nanjing China
12/2012	American Society of Cell Biology, San Francisco, CA

2013:

01/2013	**Plenary Lecture,** Asian Clinical Congress, Bangkok, Thailand (attended)
03/2013	"Building a Centrosome" Workshop, West Sussex, U.K. (attended)
05/2013	University of Algarve, Portugal (attended)
05/2013	University of Toronto, Toronto Canada (attended)
11/2013	"Anti-Cancer Drugs", Stockholm, Sweden (scheduled)
12/2013	"Anti-cancer Drugs "Moscow Russia" (scheduled)

2014:

05/2014	Massachusetts General Hospital, MGH Department of Medicine (scheduled)
03/2014	World Congress on Cell Research: Cancer Stem cells, Chicago, USA (scheduled)

9. OTHER ACHIEVEMENTS: This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

1. Based in part on the work in this proposal, a postdoctoral fellow in the laboratory, Heidi Hehnly, received an NIH K99 award. She moved to Washington University in Seattle to complete her last year of postdoctoral training on the K99 award. She will work with an outstanding scientist, Dr. John Scott, a Howard Hughes investigator. After this training, she will take an Assistant Professor position at Syracuse University in New York State.

2. A graduate student in my lab, Dr. Chun-Ting Chen, successfully obtained a postdoctoral position at the acclaimed, Scripps Research Institute in San Diego with an outstanding researcher, Dr. Chris Kintner.

3. Another graduate student in my lab, Dr. Tse-Chun Kuo, obtained an excellent postdoctoral position in Taiwan.

10. REFERENCES: List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

Kuo et al., Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. Nature Cell Biology 13: 1467, 2011. PMID: 21909099; PMCID: PMC4208311

Dyall et al., Cancer stem cells and epithelial ovarian cancer. J Oncology 1-9, 2010.

Ponnusamy et al., Ovarian cancer: Emerging concept on cancer stem cells. J Ovarian Res 86, 1-9, 2008

11. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

1 original copy of a journal article published by the Doxsey laboratory: Kuo et al, 2011

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Author Manuscript
Nat Cell Biol. Author manuscript; available in PMC 2014 October 24.

Published in final edited form as:
Nat Cell Biol. ; 13(10): 1214–1223. doi:10.1038/ncb2332.

Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity

Tse-Chun Kuo[1,9], **Chun-Ting Chen**[1,9], **Desiree Baron**[1], **Tamer T. Onder**[2,3,4,5], **Sabine Loewer**[2,3,4,5], **Sandra Almeida**[6], **Cara Weismann**[1,6], **Ping Xu**[1], **Jean-Marie Houghton**[7], **Fen-Biao Gao**[6], **George Q. Daley**[2,3,4,5,8], and **Stephen Doxsey**[1,10]

[1]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA

[2]Stem Cell Program, Children's Hospital Boston, Boston, Massachusetts 02115, USA

[3]Stem Cell Transplantation Program, Division of Pediatric Hematology and Oncology, Manton Center for Orphan Disease Research, Children's Hospital Boston and Dana Farber Cancer Institute, Boston, Massachusetts 02115, USA

[4]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA

[5]Harvard Stem Cell Institute, Cambridge, Massachusetts 02138, USA

[6]Departments of Neurology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA

[7]Department of Pathology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA

[8]Howard Hughes Medical Institute, Chevy Chase, Maryland 20815, USA

Abstract

The midbody (MB) is a singular organelle formed between daughter cells during cytokinesis and required for their final separation. MBs persist in cells long after division as midbody derivatives (MB^ds), but their fate is unclear. Here we show that MB^ds are inherited asymmetrically by the daughter cell with the older centrosome. They selectively accumulate in stem cells, induced pluripotent stem cells (iPSCs) and potential cancer 'stem cells' (CSCs) *in vivo* and *in vitro*. MB^d loss accompanies stem cell differentiation, and involves autophagic degradation mediated by binding of the autophagic receptor, NBR1, to the MB protein Cep55. Differentiating cells and

[10]Correspondence should be addressed to S.J.D. (stephen.doxsey@umassmed.edu).
[9]These authors contributed equally to this work

AUTHOR CONTRIBUTIONS

C-T.C. and S.J.D. conceived the project and wrote the manuscript with the help of T-C.K. and D.M.B. The experiments on the inheritance and localization of MB^ds as well as some for MB^d degradation were conducted by C-T.C. The experiments on MB^d accumulation were conducted by C-T.C. with the help of T-C.K. and C.M.W. Investigation of the mechanisms for MB^d degradation was conceived by T-C.K. and S.J.D., and much of the work executed by T-C.K. Autophagic flux assay, soft-agar assay of FACS-isolated cells, and MB^d localization in neural progenitors were conducted by D.M.B., who contributed substantially to the work and intellectual input on multiple aspects of this project. The reprogramming assay was conducted and analyzed by T-C.K., T.T.O, and S.L. The preparation of hESCs for live-imaging was conducted by S.A. Tissue preparation was assisted by P.X. and J.M.H.

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normal dividing cells do not accumulate MB[d]s and possess high autophagic activity. Stem cells and cancer cells accumulate MB[d]s by evading autophagosome encapsulation and exhibit low autophagic activity. MB[d] enrichment enhances reprogramming to iPSCs and increases *in vitro* tumorigenicity of cancer cells. These results suggest unexpected roles for MB[d]s in stem cells and CSCs.

INTRODUCTION

Cell division culminates in the separation of two genetically identical daughter cells[1]. During division, cell fate determinants segregate asymmetrically to stem cell progeny[2]. The two spindle poles organized by differentially-aged centrosomes contribute to this asymmetry[2,3] in that the older centrosome is inherited by the daughter cell that retains the stem cell fate[4-6].

Abscission completes cell division by severing the intercellular bridge between the two future daughter cells[1,7]. Within the intercellular bridge lies the midbody (MB), a large proteinaceous organelle[7-10] that was previously thought to detach from cells and disintegrate extracellularly as a remnant[7,8]. Recent studies show that post-abscission MBs or MB derivatives (MB[d]s) can be retained by daughter cells, suggesting alternative fates for these organelles[9,11,12].

The fate and function of MB[d]s is unclear. In neural progenitors, MB[d]s possess the putative stem cell marker CD133/prominin-1 and are proposed to participate in intercellular signaling during neural development[13,14]. MB[d]s can be degraded by autophagy (see below)[12], but the relationship between MB[d] loss or retention and the physiological state of cells is unknown.

During autophagy (macroautophagy), double membrane-bound autophagosomes assemble, engulf cytoplasmic material, and fuse with lysosomes for degradation[15-18]. Autophagy is required for cellular homeostasis, eliminating defective ubiquitin-tagged proteins and organelles[16-19], clearing cell fate determinants and cell remodeling[20-22]. Defects in autophagy contribute to many disorders, including neurodegeneration[23], hepatomegaly[24] and aging[15,18].

Here we show that MB[d]s accumulate in stem cells and are lost upon differentiation. They are selectively degraded by linking the NBR1 autophagic receptor to the Cep55 MB protein. MB[d]s accumulate by evasion of autophagosome encapsulation, asymmetric inheritance, and maintenance of low autophagic activity. Reprogramming efficiency and *in vitro* tumorigenicity are increased following experimental elevation of MB[d] levels suggesting non-mitotic roles for these organelles in stem and cancer cells.

RESULTS

Post-mitotic midbodies accumulate within cells

Multiple MB[d]s were observed in subpopulations of cells by immunofluorescence (IF), but their precise location was unclear (up to 20; Fig. 1a, b). Three-dimensional reconstruction of immunofluorescent images revealed multiple MB[d]s inside polarized and nonpolarized cells

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(Fig. 1c, d). Immuno-electron microscopy confirmed this localization and revealed ultrastructural features characteristic of MB^{d}s[8,14] (Fig. 1e). About 70% of cell-associated MB^{d}s were trypsin-resistant, suggesting that they were intracellular (Fig. 1f). This intracellular localization of MB^{d}s suggested that they might accumulate in cells through successive divisions (below).

MB^{d}s were also released from cells. In 2-day co-cultures of HeLa cells stably expressing either monomeric RFP (cytoplasmic marker) or MKLP1-GFP (MB marker), about 7% of MKLP1-GFP+ MB^{d}s associated with RFP+ cells (Fig. 1g). Such free MB^{d}s were also generated by other cell types (e.g. human adult fibroblasts, HeLa; 1-10%). These observations resolve the conflict of previous studies suggesting that MB^{d}s are either retained and degraded[9,11,12] or released as remnants after abscission[8]. We show that MB^{d}s accumulate in some cells (Fig. 1a-d) but not others, and it is this cell type-specific difference in MB^d-accumulation that is the focus of this study.

MB^{d}s are inherited by the cell with the older centrosome

Multiple MB^{d}s often clustered around the centrosome or spindle pole (ref. 9 and data not shown), reminiscent of MB^d-sized aggresomes, which segregate to one daughter cell under control of centrosomes[25,26]. Moreover, centrosome age-dependent differences in signaling were observed late in cytokinesis[27]. These centrosome age-related differences led us to examine the relationship between centrosomes and MB^d inheritance.

In G1, the centrosome contains one mother centriole (MC) and one daughter centriole (DC)[3]. After centriole duplication, three generations of centrioles are present: an older mother, a younger mother and two new daughters[3,27]. The centrosome with the older MC is termed the older centrosome[4,5]. GFP-tagged centrin1 (CETN1-GFP)[28] expressed in mitotic HeLa cells was brightest at one of the four centrioles (92.2% of cells, n=116; Fig. 2a) and turned over very slowly (FRAP $t_{1/2}$ ~4 hours and ref. 5). The brightest centriole remained so from metaphase to late cytokinesis (91.3% of cells, n=46; supplementary information, Fig. S1a), suggesting that it was the older MC. This was confirmed by staining with the older centrosome marker, hCenexin1[27] (~90% of HeLa and MCF-7 cells, n=143 and n=347, respectively; Fig. 2b). Several other centriole antigens also showed intrinsic age-related differences in labeling (supplementary information, Fig. S1b).

Using CETN1-GFP to identify the older MC; bright-field imaging to follow MB dynamics in living cells; and immunofluorescence to confirm MB^d inheritance, we determined that MB^{d}s were preferentially inherited by the cell with the older centrosome. This was observed in pluripotent human embryonic stem cells (hESCs; 83.3% of H9, n=18; Fig. 2d), immortalized somatic cells (91.3% of hRPE-1, n=23) and cancer cells (U2OS: 84.6%, n=13; HeLa: 75.0%, n=24; Fig. 2c). We conclude that most inherited MB^{d}s are asymmetrically transferred to the daughter cell with the older centrosome in several cell types.

MB^{d}s accumulate in stem cells *in vivo*

Other studies have shown that the older centrosome is asymmetrically inherited by the stem cell during asymmetric divisions in the *Drosophila* male germline[4] and the mouse neocortex[5]. The association of the older centrosome with both MB^{d}s and stem cell divisions

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led us to ask whether MB^{d}s were found in stem cell niches. To address this, we determined the localization of MB^{d}s in human and mouse tissues. In seminiferous tubules of testes, MB^{d}s were confined to the basal compartment, the site of germline stem cells and their mitotic progeny (both capable of self-renewal[29,30]) (Fig. 3a, up to 8 puncta/cell, 5-μm section). Electron microscopy also revealed multiple cytoplasmic structures with features characteristic of MB^{d}s within these cells (Fig. 3b, c).

In the ventricular zone (VZ, Sox2+[31]) of embryonic mouse brains, CD133-labeled MB^{d}s were associated with neural progenitors[13,14] (Fig. 3d and Supplementary information, Fig. S2). During asymmetric divisions, intracellular MB^{d}s were usually found in ventricle-facing daughter cells (progenitors; 75%, n=8) and not in daughters with presumed committed fates[5]. MB^{d}s in the human hair follicle were also confined to a subpopulation of cells in the stem cell niche, the bulge[32], suggesting distinct properties of this subpopulation (Fig. 3e, f). MB^{d}s were also enriched in β1–integrin+[33] mouse skeletal muscle progenitors (SMPs; 4-fold) over non-SMP cells. These observations suggested that MB^{d}s were selectively retained and accumulated during successive stem cell divisions *in vivo*.

MB^{d}s accumulate in stem cells *in vitro*

To rigorously test the idea that MB^{d}s are selectively inherited by stem cells, we examined MB^d fate during stem cell differentiation and somatic cell reprogramming. MB^d 'accumulation' was assessed by counting cells with >1 MB^d, as all cells can transiently acquire one MB^d after abscission (below). MB^d-accumulation decreased ~8-fold upon differentiation of hESCs (H1-OGN) to fibroblast-like cells (dH1f; Fig. 3g, h). Differentiation was judged by loss of embryonic stem cell markers (Oct4, Sox2, Klf4, Nanog) and gain of the CD13 differentiation marker[34,35]. In contrast, MB^d-accumulation increased ~7-fold after reprogramming dH1f cells to iPSCs[34,36] (dH1f-iPS; Fig. 3h, i). We conclude that MB^d-accumulation *in vitro* reflects that observed *in vivo*, and can be manipulated by altering the potency status of cells.

MB^d-accumulation is enhanced in tumor-derived cells

We next examined differences in MB^d-accumulation among cell lines derived from stem cells, normal dividing cells and cancer cells (Fig. 4a). MB^d-accumulation was low in primary and telomerase-immortalized normal cells and significantly higher in hESCs and iPSCs (~7-fold on average; Fig. 4a). Most cancer cells exhibited even higher levels of MB^d-accumulation. For example, MB^d-accumulation in tumorigenic MCF-10AT and MCF-10CA1a cells was much higher than in the normal MCF-10A parental line. The common ability of stem cells and cancer cells to accumulate MB^{d}s, express stem cell markers[37] and possess stem cell properties[38,39] suggests a relationship between MB^d-accumulation, tumorigenicity and cancer 'initiating' or 'stem' cells defined by the CSC theory[40].

MB^d-accumulation does not correlate with cell proliferation rate

A simple explanation for cell type-specific differences in MB^d-accumulation is variability in proliferation rates. Slower division rates could allow more time for MB^d degradation, as recently proposed[12]. However, we observed no correlation between population doubling-

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time and MB^d-accumulation (Fig. 4a). It was still possible that MB^d-accumulating cells cycled faster than the bulk population. However, a cohort of cells pulse-labeled with EdU[41] showed a proportional decrease in EdU intensity, reflecting dilution of dye after successive divisions (Fig. 4b) and indicating that MB^d-accumulating and non-accumulating subpopulations had similar cycling rates (Fig. 4c, d).

MB^d-accumulating cells evade membrane encapsulation of MB^{d}s

We next asked if MB^{d}s occupied different sites within MB^d-rich and MB^d-poor cells. To test this, we used the Fluorescence Protease Protection (FPP) assay[42] to monitor degradation of MB^{d}s following plasma membrane permeabilization and protease addition (Fig. 5a). Under these conditions, MKLP1-GFP+ MB^{d}s were degraded in MB^d-rich HeLa cells but not in MB^d-poor hRPE-1 cells indicating that MB^d-poor cells sequestered MB^{d}s in membrane-bound compartments whereas MB^d-rich cells accumulated them in the cytoplasm (Fig. 5b). Importantly, the integrity of intracellular organelles was maintained during the course of these experiments (supplementary information, Fig. S3).

Stem cells and cancer cells evade lysosomal degradation of MB^{d}s

The protease resistance of MB^{d}s and low MB^d-accumulation in MB^d-poor hRPE-1 cells (Fig. 4a and 5b) suggested that MB^{d}s were delivered to a membrane-bound compartment for degradation, such as the lysosome. Indeed, MB^{d}s were often found within LAMP2[43]-labeled lysosomes in MB^d-poor cells (Fig. 5c). To test this further, we examined the fate of newly-formed MB^{d}s in synchronous populations of MB^d-poor cells (Fig. 5d). Three hours after release from mitosis, the percent of MB^d+ cells (MB^d levels) peaked at ~40% (50% being the maximum since half the cells were 'born' without a MB^d). This was followed by a peak in MB^d localization to lysosomes (~42% at 7 hours; Fig. 5d) and then a decrease of MB^{d}s to baseline levels (16-19 hours; Fig. 5d). These data and the FPP data suggested that MB^{d}s in hRPE-1 cells entered the cytoplasm, moved into lysosomes and were degraded before the next cell cycle (Fig. 5b, d).

If lysosomes are involved in MB^d degradation, lysosomal inhibition should increase MB^d levels. Indeed, when lysosomal activity was inhibited in MB^d-poor hRPE-1 cells with either chloroquine or E64d/PepA protease inhibitors[44] MB^d levels (Fig. 5e) and the percent of MB^{d}s found within lysosomes (Fig. 5c) were elevated. In contrast, MB^d levels and the percent of MB^{d}s in lysosomes in MB^d-rich cells (hESC, MCF-7; Fig. 5c, e) were largely unaffected by lysosomal inhibition (see supplementary information, Fig. S4a). The modest increase in MB^d+ HeLa cells (Fig. 5e) was consistent with their modest MB^d-accumulating ability (Fig. 4a). We conclude that lysosomal degradation prevents MB^d-accumulation in MB^d-poor cells, but does not play a major role in MB^d-rich cells (e.g. stem cells, CSCs) thus allowing MB^{d}s to accumulate.

Autophagic degradation controls intracellular MB^d levels

To determine how MB^{d}s were directed to lysosomes, we explored pathways leading to lysosomal degradation. Reported autophagy levels in MCF-7 and DLD-1 cells[45,46] suggested a relationship between autophagy and MB^d fate. Low autophagy levels in MCF-7 cells resulting from a deficiency in the autophagy gene, *BECN1* (also known as *Atg6*)[45], are

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consistent with high MB^d-accumulation (~26-fold over normal cells; Fig. 4a). High autophagy levels in DLD-1 cells[46] are consistent with low MB^d-accumulation (only ~1.8-fold over normal cells; Fig. 4a). In agreement with this trend was the presence of MB^ds in autophagosomes of MB^d-poor cells (Fig. 6a).

Experimental reduction of autophagy activity using MEFs from *Atg5*-deleted mice[19] or by siRNA-mediated depletion of Atg7, increased MB^d levels (Fig. 6b). Induction of autophagy by rapamycin and lithilum chloride treatment[47,48] in HeLa cells or by exogenous BECN1 expression in MCF-7 cells, decreased MB^d levels (Fig. 6c). These results demonstrated the role of autophagy in regulating MB^d levels in different cell types, and suggested an inverse relationship between autophagic activity and MB^d-accumulation. This inverse relationship was revealed in 12 cell lines by LC3-II[44,49] or p62[44,50,51]-based measurements of autophagic activity (Fig. 6d, e and supplementary information Fig. S4b). We conclude that MB^d levels are, in part, modulated by cell type/lineage-specific autophagy (Fig. 3g-i, 4a, 6d and 6e).

NBR1 is an autophagic receptor for MB^d-specific degradation

To test whether MB^d degradation involves non-specific or receptor-mediated autophagy pathways[15], we investigated the mammalian autophagic receptors, p62[50-52] and NBR1[53,54]. p62 is implicated in MB^d clearance[12], whereas NBR1 is untested. NBR1 and p62 localized to mitotic MBs and MB^ds (Fig. 7a, top, data not shown, and ref. 12), suggesting that MB^d degradation involves receptor-mediated autophagy. NBR1-silencing in HeLa cells increased MB^d levels to Atg7-silencing levels (Fig. 6b and 7b), suggesting that NBR1 is likely a major autophagic receptor for MB^d degradation. In contrast, *p62*-deletion[51] or siRNA-mediated p62 depletion had no detectable effect on MB^d levels (Fig. 7b, c) or NBR1 recruitment to MB^ds (Fig. 7a, bottom).

To date, no MB^d target(s) for autophagic degradation have been identified. Candidate-based screening revealed that endogenous NBR1 co-immunoprecipitated with the MB protein Cep55 in hRPE-1 cells (Fig. 7d). Cep55 over-expression increased MB^d levels (Fig. 7e) and the level of NBR1-negative MB^ds (Fig. 7f), presumably through NBR1 sequestration in the cytoplasm (Fig. 7g). This suggested a role of Cep55 in NBR1-mediated MB^d degradation. We propose that the Cep55/NBR1 interaction couples MB^ds to the autophagic machinery to control MB^d fate.

Cells enriched in MB^ds exhibit increased reprogramming efficiency

We next examined the functional consequences of manipulating MB^d levels. We first tested the role of MB^ds during reprogramming[34,35,55] in cells stably expressing NBR1-specific shRNAs (shNBR1) to increase MB^d levels over controls (shNT). MB^d levels increased ~1.8-fold in dH1f cells, ~1.5-fold in IMR90[55] embryonic fibroblasts, and ~1.9-fold in hFib2[34] adult fibroblasts. Under these conditions, iPSC colony formation increased significantly in all three cell types depleted of NBR1: dH1f cells (up to 8.7-fold, avg. 3.1±0.5-fold), IMR90 cells (up to 4.2-fold, avg. 3.4±0.8-fold; Fig. 8a, b and supplementary information Table. S1) and adult hFib2 cells (up to 2.5-fold, avg. 1.7±0.5-fold). Similar results were obtained with different batches of viruses, different combinations of reprogramming factors, and different

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viral delivery systems (see Methods). Importantly, increased reprogramming following NBR1-depletion occurred without significant changes in global autophagic activity (dH1f; Fig. 8c) or cell proliferation rate (shNBR1: 27.3±2.5hrs; shNT: 26.8±4.5hrs; n=6), suggesting that NBR1 is selective for MB^d degradation.

Cancer cells enriched in MB^{d}s exhibit increased *in vitro* tumorigenicity

Because MB^{d}s selectively accumulate in stem cell niches, hESCs, and iPSCs, we reasoned that they may also accumulate in CSCs. On the basis of Hoechst 33343 extrusion, the side population (SP) of MCF-7 cells[56] was isolated. These putative CSCs showed a 7-fold increase in MB^d+ cells over the non-SP population (MP; Fig. 8d).

To directly address the role of MB^{d}s in cancer cells, MKLP1-GFP-expressing HeLa populations with high or low percentages of MB^d+ cells were isolated by FACS, and tested for anchorage-independent growth. Increased colony formation was observed in the "MB^d high" versus the "MB^d low" population, and colony formation increased with increasing MB^d levels (up to 4-fold; Fig. 8e). An increase in colony formation was also observed in MB^d-enriched HeLa cells (Fig. 8f, left) and mouse hepatocarcinoma cells (134-4; Fig. 8f, right) following NBR1-silencing. Results of all three strategies suggest that MB^{d}s in cancer cell subpopulations may contribute to their tumorigenic potential.

DISCUSSION

We have identified new roles for MB^{d}s outside their canonical function in cytokinesis. This work provides the first evidence for MB^d-accumulation in stem cells, hESCs and iPSCs *in vivo* and *in vitro*, and for dramatic MB^d reduction in differentiating progeny of stem cells. MB^{d}s appear to function in maintaining or enhancing the pluripotency of stem cells and the tumorigenicity of cancer cells.

Our findings suggest that MB^d loss that accompanies stem cell differentiation is mediated by autophagic degradation, resulting in selective elimination of MB^{d}s in differentiated cells but retention in germ or stem cells. This process is intriguingly similar to clearance of P granule components in committed somatic cells of *C. elegans*, which is also mediated by autophagy[57]. Moreover, P granules contain molecules required for cell fate specification[58], and MB^{d}s contain stem cell markers[13,14] and enhance cell fate conversion (present study). It is thus tempting to propose that MB^{d}s may serve as scaffolds for organizing cell fate determinants. Equally intriguing is the observation that essentially all cancer cells examined contain MB^d-accumulating subpopulations, making this a common intrinsic property of both stem cells and cancer cells. The observation that MB^d-enriched cancer subpopulations exhibit enhanced *in vitro* tumorigenicity is consistent with the CSC model for potentiation of tumorigenicity[37-40].

Our data identify two primary mechanisms for MB^d-accumulation. The first is asymmetric MB^d inheritance by the daughter cell with the older centrosome (Fig. 8g, top). In fly testes and mouse neocortex, the old centrosome segregates to the stem cell during asymmetric divisions and is accompanied by increased microtubule-anchoring ability[4-6]. MB^d inheritance could be facilitated through increased anchoring of microtubules to the older

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centrosome, and increased microtubule binding to the MB^d in the daughter cell with the older centrosome. This would be consistent with the observed MB^d-accumulation in stem cells but not in their differentiated progeny. Despite the slower division rate of stem cells *in vivo*[59], MB^d-accumulation could still occur via this mechanism. However, our results also indicate that such asymmetry occurs in different cell types, suggesting that it may only be physiologically relevant in stem cells and CSCs.

Evasion of autophagic degradation is a second mechanism for MB^d-accumulation (Fig. 8g, bottom). This is exemplified by the inverse relationship between MB^d levels and autophagic activity, and by changes in MB^d levels with manipulation of autophagy levels. MB^d-accumulation can also be mediated by uncoupling receptor-mediated entry into the autophagy pathway, since depletion of the NBR1 autophagic receptor or over-expression of the corresponding ligand, Cep55, increases MB^d levels. In contrast, another known autophagic receptor, p62, does not appear to be involved in MB^d clearance (Fig. 7b, c). NBR1 and p62 can form a complex[53,60]; however, evidence suggests that they may act independently as autophagic receptors[53]. Thus, p62/NBR1 complex formation may not be a prerequisite for autophagic degradation. Since NBR1-silencing increases MB^ds to levels seen following inhibition of autophagy in HeLa cells (Fig. 6b and 7b), NBR1-mediated autophagic degradation likely represents a major pathway for selective MB^d elimination. However, it is still possible that other autophagic receptors and MB^d ligands may exist and contribute to MB^d degradation, even though Cep55 is the sole MB ligand for the NBR1 receptor identified thus far (Fig. 7d). In our model, Cep55 and NBR1 and perhaps other MB^d ligands and autophagy receptors, act as switches that control MB^d fate. Ongoing proteomic analyses may identify other molecules and pathways for MB^d degradation.

MB^d levels can be further increased in autophagy-compromised *Atg5*$^{-/-}$ MEFs when lysosome enzymes are inhibited (data not shown), suggesting that other degradative pathways may contribute to MB^d degradation. Chaperone-mediated autophagy (CMA)[15,61], which targets ~30% of cytosolic proteins and is upregulated upon compromised autophagy[62], is a potential candidate since multiple MB proteins contain CMA-targeting motifs (KFERQ-like motifs)[61]. The proteasome system is another major cellular degradation pathway[63] but it doesn't appear to play a role in MB^d degradation (supplementary information, Fig. S5).

Other non-degradative processes may also regulate MB^d levels. Even though elevated proliferation rate has been proposed as a factor hindering autophagic MB^d degradation and causing MB^d-accumulation in cancer and normal cells[12], we didn't observe such a correlation (Fig. 4a). Additional work is required to determine if MB^d-accumulation also requires selective sequestration of previously inherited (pre-existing) MB^ds, as suggested by selective accumulation of MB^ds in stem cells of the testes and lateral ventricle of the brain (Fig. 3a-d). Release of MB^ds has also been observed in chicken and mouse neural progenitors[13,14] and in human cells (ref. 8 and Fig. 1g), and may be another, possibly minor pathway for eliminating MB^ds (or for intercellular signaling[14]). Finally, ongoing work is addressing whether MB^ds are distributed to both daughters of stem cells during symmetric divisions as might be expected if MB^ds are essential for stem cell function.

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In summary, our results demonstrate that MB^ds are more than the remnants of cytokinesis. Their fate is differentially controlled in different cell types and mediated by diverse pathways. The shared ability to accumulate MB^ds by stem cells and putative CSCs, and the striking impact on cellular phenotypes following manipulation of MB^d levels suggest that MB^ds perform important cell type-specific functions that remain to be discovered.

METHODS

Cell lines

hESC and iPSC lines include H1 (WA01), H9 (WA09), H1-OGN (Oct4-EGFP knock-in H1)[36], and dH1f-iPS[34], which is reprogrammed from dH1f cells differentiated from H1-OGN (HSCI at Children's Hospital Boston). Differentiated lines include hRPE-1 (Clontech), MCF-10A, adult human fibroblasts (PCS-201-012, ATCC), hFib2[34], IMR90 (CCL-186, ATCC), *ex vivo* C57BL/6 MEFs, GFP-LC3-expressing $Atg5^{-/-}$ and $Atg5^{+/+}$ MEFs[19], and $p62^{-/-}$ and $p62^{+/+}$ MEFs[51]. Cancer cell lines include DLD-1, HeLa, NCC-IT, PC-3, U2OS, SAOS-2, 134-4, MCF-7, MCF-10AT, and MCF-10CA1a. Mouse skeletal muscle progenitors (SMPs)[33] and *in vitro* activated T cells were isolated and stimulated following standard protocols. Cells were used within 4 (primary cultures) or 10 (established cell lines, hESCs, and iPSCs) passages. Cells expressing MKLP1-GFP, monomeric RFP and CETN1-GFP were created in the present study or ref. 28.

Immunofluorescence and Immunohistochemistry

Immunofluorescence was performed as described[9,13,64]. To label lysosomes and autophagosomes, cells were permeabilized with 0.05% saponin in blocking buffer (10% goat serum/PBS). Preparations for immunohistochemistry were fixed with 4% paraformaldehyde/0.5% glutaraldehyde via perfusion. Testes were processed and stained following 2-4hr post-fixation with 4% paraformaldehyde. MB-derived rings between spermatocyte syncytia[65] were observed if stained longer. Images were taken on a Zeiss Axioskop 2 microscope, a Zeiss Axiovert 200 microscope with PerkinElmer UltraView LAS spinning disc, or an Olympus BX-51 microscope. Images were processed and analyzed with MetaMorph (Molecular Devices) and Imaris (Bitplane Inc.).

Electron Microscopy

Conventional EM—Mouse tissue, fixed with 5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH=7.4) for 30 min via perfusion, was diced into 1-mm cubes for 1-hr post-fixation at 4°C. Cubes were washed with cacodylate buffer, stained and embedded in Spi-pon/Araldite, and sectioned at 70-500 nm before staining with 25% uranyl acetate and Reynold's lead citrate. Images were taken on a Philips CM12 electron microscope with an Erlangshen CCD Camera (Gatan).

Immunogold EM—MCF-7 cells on coverslips were prepermeabilized for 60 sec with preperm buffer (80 mM PIPES, pH6.8, 0.5 mM EGTA, 1 mM MgCl, 0.5% Triton X-100), fixed with 4% paraformaldehyde for 10 min, labeled for MKLP1 for 1 hour, processed as described[66] using 12-nm gold-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) and embedded in Spi-pon/Araldite. 80-nm sections were cut, stained and viewed as above.

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Time-lapse imaging

CETN1-GFP-expressing lines were grown on 35-mm MatTek dishes (MatTek Corp.) or coverslips before imaging[9]. H9 hESCs were seeded on matrigel-coated dishes overnight, then transduced with CETN1-GFP, and grown for >72 hours in complete mTeSR1 medium (Stemcell Technologies). The transduced cells were imaged every 15 min in phenol red-free D-MEM/F12 medium (Invitrogen) with mTeSR1 supplement and 10 mM HEPES, and stained to confirm MB^d inheritance. Duplicate dishes of transduced cells were stained for stem cell markers to ensure cell quality.

MB^d quantification

Quantification was based on the markers that: 1) labeled both mitotic MBs and MB^ds (MKLP1, mgcRACGAP, or Cep55); 2) labeled MBs differently than MB^ds (α-tubulin or Aurora B); 3) defined cell boundaries (α-tubulin or ZO-1). Because Cep55, MKLP1, and mgcRACGAP also label centrioles and spindle midzones, cells were co-stained with centrosome antibody (e.g. 5051), and a size threshold for MB/MB^ds (1 μm) was introduced to exclude non-MB^d structures. Structures with MB-specific or non-MB/MB^d labeling were excluded from MB^d counts. Cell counts: For hESCs, 5-11 colonies were imaged from triplicates in each experiment. For other cell types, random fields were imaged until n > 500 cells. Each dividing cell was considered one cell.

Doubling time calculations

Cells were seeded (1-1.5×10^5/60-mm dish), and total cell counts were taken by hemocytometer every 24 hours for 4 days. Alternatively, cells were seeded (2.5-5.0×10^3/ well, 96-well plates), and the absorbance from an MTS-based colorimetric assay (#G3582; Promega Corp.) was used to estimate cell counts every 24 hours. Timepoints vs. Log_{10}(avg. cell counts or absorbance at that timepoint) was plotted and the slope ascertained. $T_{1/2}$ = $Log_{10}(2)$/ slope. For some cell lines, both methods were used and gave similar results.

MB^d localization assays

Extracellular trypsin treatment—MKLP1-GFP-expressing HeLa cells grown in MatTek dishes were imaged every 3 min, and underwent no morphological changes upon replacement of media with PBS. After trypsin addition, GFP+ MB^ds were monitored for 60-90 min for intensity reduction (degradation) or detachment from cells (dissociation).

Co-culture assay—Equal numbers of monomeric RFP- or MKLP-GFP-expressing cells were seeded and co-cultured in 60-mm dishes with coverslips. Cells were stained 2 days later, and the percentage of GFP+ MB^ds associated with RFP+ cells was determined.

FPP assay—The FPP assay was carried out as reported[42] except cells were plated in MatTek dishes 24 hours before co-transfection of MKLP1-GFP and GAPDH-dsRed (Lipofectamine 2000, Invitrogen). Cells were permeabilized and then digested with proteinase K (50 μg ml^{-1}). Constructs labeling mitochondria, peroxisomes, ER and Golgi were used as controls.

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Lysosome and proteasome assays

Cells at 70% confluency were incubated with chloroquine (200 μM/PBS; Sigma), E64d + pepstatin A (E64d/PepA) (10 μg ml^{-1}/DMSO each; Sigma)[44,51] or solvents alone (controls) for 22 hours before fixation. Lysosome inhibition was confirmed and visualized after 12-hour DQ-Red BSA (10 μl ml^{-1}; Invitrogen) incubation. Mitotic hRPE-1 cells were treated with proteasome inhibitors, MG132 (1 μM; Sigma) or lactacystin (50 μM; Sigma) 1 hour after replating.

Autophagy manipulation assays

MB^ds were quantified in >500 cells in triplicate unless otherwise noted.

Protein depletion—siRNAs targeting human Atg7[67], p62[12], NBR1[53] (2503-2521 bp, GenBank NM 005899), Lamin A/C[9], and GFP (5'-NNCAUGAAGCAGCACGACUUC-3') were Dharmacon. MB^d levels were analyzed 48 hours after 1-nmol siRNA transfection (Oligofectamine, Invitrogen). For NBR1 and p62 experiments, only cells negative for p62 and/or NBR1 immunofluorescence were analyzed.

Beclin1 (BECN1) overexpression—MB^d levels were analyzed in 265 Flag+ and 2200 control MCF-7 cells 48 hours after Flag-BECN1 (4 μg) or mock nucleofection (Amaxa).

LiCl + rapamycin treatment—MB^d levels in HeLa cells were examined 24 hours after treatment with LiCl (10 mM; Sigma) and rapamycin (200 nM; Calbiochem), or with DMSO.

CEP55-EGFP overexpression—MB^d levels and its NBR1-association were assessed in hRPE-1 cells (1×10^5/well, 6-well plates) 48 hours after CEP55-EGFP (1 μg), EGFP (1 μg) or mock transfection.

Biochemical assays

Protease and phosphatase inhibitors, cell lysates, SDS-PAGE and immunoblotting were purchased or carried out as described[9] unless specified.

Autophagy flux determination—Lysates of E64d/PepA (I) and DMSO (U) treated cells were blotted for α-tubulin and LC3. LC3-II levels were determined and normalized to α-tubulin using ImageJ. Autophagic flux = |100 – ((U/ I LC3-II level) × 100)|.

Immunoprecipitation—hRPE-1 cell lysates (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 4°C) were pre-cleared for 1 hour with protein G-plus conjugated agarose beads (Santa Cruz) at 4°C, incubated with 2 μg normal IgG, anti-Cep55 or anti-NBR1 antibodies for 3 hours at 4°C, and incubated overnight at 4°C with 25 μl protein G-plus beads. Following washes with lysis buffer and elution, immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting.

Assays for MB^d function

Cellular reprogramming—Viral production, transduction and reprogramming were performed as described[34,35,55,68]. Commercially-available shRNA against NBR1 (pSM2c-

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shNBR1, V2MM_36901; 4-22 bp, GenBank NM 005899) was cloned into pGIPZ lentiviral vector (Open Biosystems). Embryonic fibroblasts (IMR90), adult fibroblasts (hFib2) and dH1f cells were transduced with either NBR1-specfic or non-targeting shRNA vector, and puromycin-selected to establish NBR1-depleted (shNBR1) and control (shNT) lines. dH1f (2.5×10^4/assay) were reprogrammed with lentiviral vectors[69] (Addgene #21162 and 21164) expressing *OCT4, SOX2, KLF4* and *c-MYC*[34,35,68] whereas the reprogramming of IMR90 and hFib2 cells (5×10^4/assay) also included lentiviral vectors expressing Nanog and Lin28[55,69] (Addgene #21163). iPSC colonies were quantified on day 21 based on Tra-1-60 expression using ImageJ, as reported[35,68], and with parameters: ≥148 (threshold), 0.5-1 (circularity), and either 10-infinity or 30-infinity (size).

Side Population (SP) assay—The assays were carried out as previously described[56] in MCF-7 cells. The MB^d levels in SP and non-SP populations were determined as described above.

Soft-agar assays—"MB^d high" and "MB^d low" subpopulations of MKLP1-GFP-expressing HeLa cells were separated by FACS, and plated in soft-agar (2.5×10^4/well, 6-well plates). The MB^d levels were determined 12-15 hours after plating aliquots of subpopulations onto coverslips. For the NBR1-silencing soft-agar assay, NBR1-depleted (shNBR1) and control (shNT) cells (1×10^5/100-mm dish) were plated. For both assays, cells were grown for ~3 weeks at 37°C, and stained as described[70]. Colonies were quantified microscopically, and the average from triplicate wells or plates presented.

Antibodies

Antibodies to the following proteins/tags were used in this study—Atg5 (1:2000, Cosmo Bio, CAC-TMD-PH-ATG); Atg7 (1:1000, ProSci, 3617); Actin (1:300, Sigma, AC-40); Aurora B (1:100, BD Trans Lab, 611082); CD13 (1:50, BioLegend, 301707); CD133 (1:200, eBioscience, 14-1331); Cep55 (1:50, 1:100 and 1:1000 for immunofluorescence, Abnova #H00055165-B01, Abnova #H00055165-A01, and the gift from K. Kurtche, respectively; 1:500 for immunoblotting, Genetax #GTX112190); hCenexin1 (1:100, a gift from K.S. Lee); Centriolin (1:200, ref. 9); Flag (1:200, Sigma, F7425); GAPDH (1:8000; Santa Cruz, SC-32233); GFP (1:1000; Abcam, ab6556 and Santa Cruz, sc-9996); GT335 (1:100; a gift from P. Denoulet); β1-Integrin (1:50; BD Phramingen); K15 (1:100; Lab Vision, MS-1068-P); LC3 (1:10 for immunofluorescence, Nano Tools, LC3-5F10; 1:300 for immunoblotting, Novus Bio NB100-2331); LAMP2 (1:50, H4B4 from DSHB); mgcRACGAP (1:500, Abcam, ab2270); MKLP1 (1:1000 for immunofluorescence, 1:200 for immunohistochemistry, 1:10 for immuno-EM, Santa Cruz, sc-867); NBR1 (1:500, Abnova, H00004077-B01P); p62, human samples (1:500, BD Trans Lab, 610833); p62, mouse samples (1:1000, Progen, GP62-C); RFP (1:200, Clontech, 632496); Na-K-ATPase (1:15, α6F from DSHB); α-tubulin (1:100 for immunofluorescence, 1:400 for immunoblotting, Sigma, T9026a; 1:100 for immunofluorescence, Millipore, CBL270); α-tubulin-FITC (1:300, Sigma, F2168); Tra-1-60-biotin (1:200, eBioscience, 13-8863); Ubiquitin (1:2000, BD BioSci, #550944); WGA-Alexa Fluor 555 (1:200, Molecular Probes, W32464); ZO-1-FITC (1:50, Zymed, 33-9111).

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Statistics

Data was analyzed by Student's one-tailed paired *t*-test or unpaired with Welch's correction unless specified. One-way ANOVA was used in conjunction with Tukey's test for comparisons among multiple groups. For the EdU-labeling assay, the EdU intensity was first logarithmically transformed for the use of one-way ANOVA. Statistically analyzed experiments were completed at least 3 times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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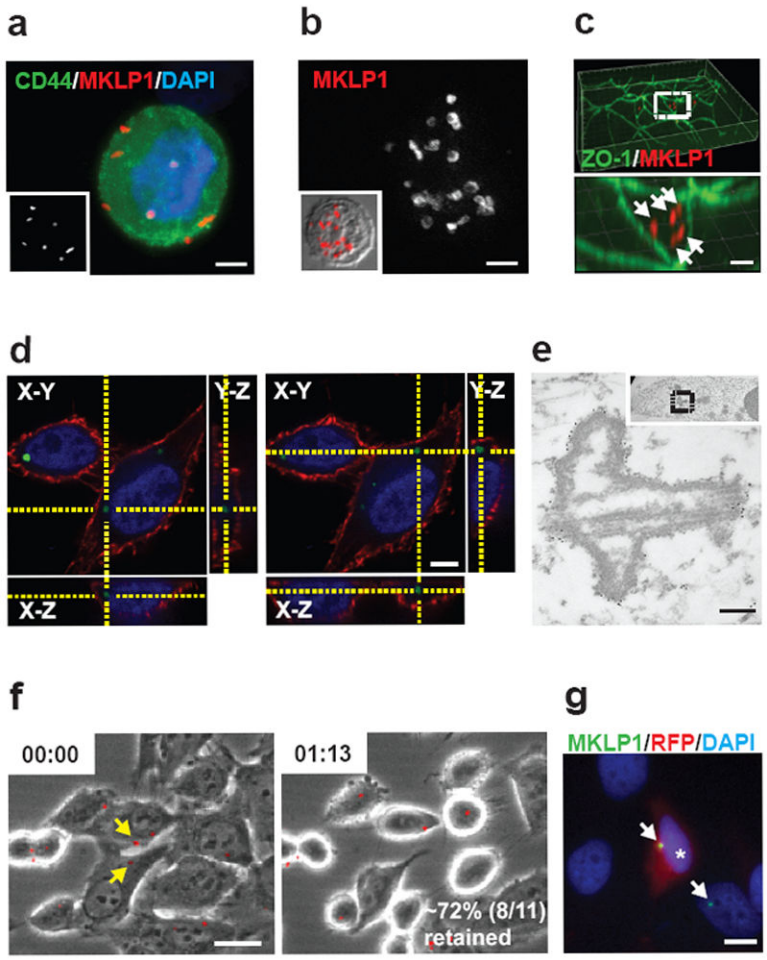

Figure 1.

MB^{d}s accumulate within cells. (**a, b**) Multiple MB^{d}s associate with a PC3 cell (a) and a B-lymphoblast (b). Insets (a) MB^d labeling and (b) merged phase-contrast image with MB^d labeling to show cell boundaries. MKLP1, MB^d marker (a, b; red); CD44, membrane (a; green); DAPI, DNA (a; blue). Bar, 5 μm (a) and 2 μm (b). (**c, d**) Three-dimensional reconstruction of polarized cells in a monolayer (c) and a HeLa cell (d) show intracellular MB^{d}s. (c) ZO-1, tight junction; MKLP1, MB^{d}s. Bar, 2 μm. Enlargement (c, bottom) of box (c, top) shows five MB^{d}s (arrows). (d) Wheat germ agglutinin, plasma membrane (red); MKLP1-GFP, MB^{d}s (green); DAPI, DNA (blue). Bar, 5 μm. (**e**) Electron micrograph of a MB^d in a permeabilized MCF-7 cell showing immungold labeling with MKLP1 antibodies. Inset, lower magnification of the MB^d (boxed) in cell; nucleus, right. Bar, 200 nm. (**f**) Time-lapse images during extracellular trypsin treatment of HeLa cells show retention of most MB^{d}s (MKLP1-GFP, red). Two MB^{d}s (yellow arrows) are lost upon treatment, suggesting digestion and/or dissociation. Time (hr:min) post-trypsin. Bar, 5 μm. (**g**) Two-day co-cultures of HeLa cell expressing either MKLP1-GFP (MB^d marker) or cytosolic RFP. Green MB^{d}s (arrows) associated with red cells (asterisk) indicate post-mitotic transfer of MB^{d}s between cells. Bar, 10 μm.

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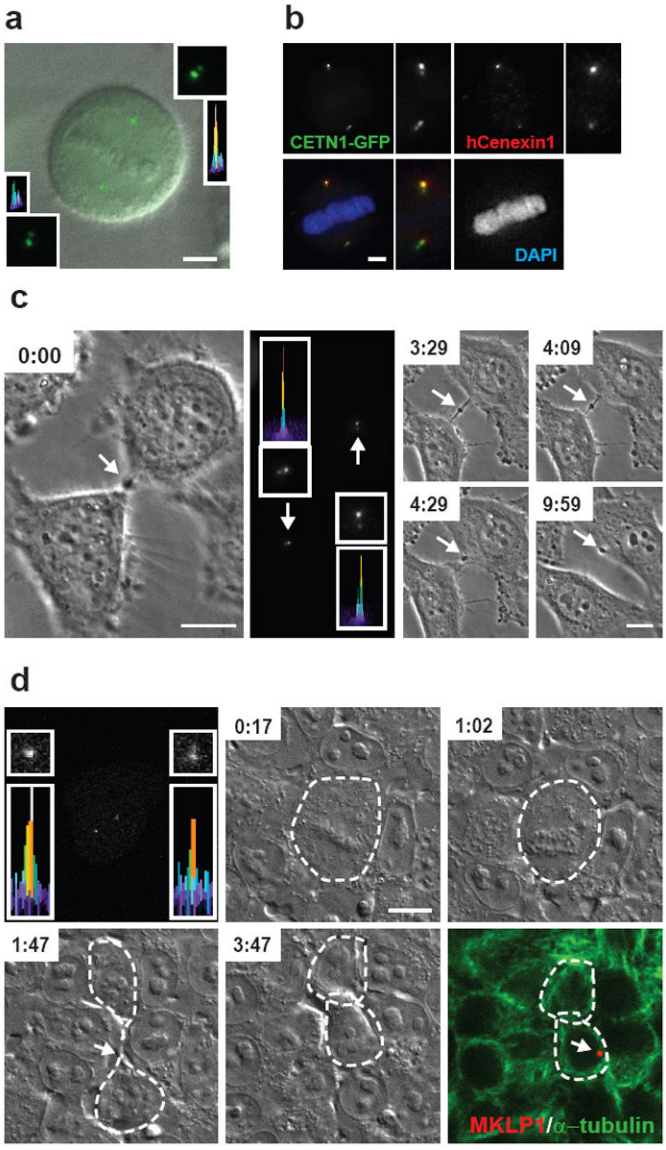

Figure 2.

MB[d]s are preferentially inherited by the cell with the older centrosome. (**a**) CETN1-GFP signal is brighter in upper centrosome/spindle pole of a mitotic spindle. The merged DIC image with CETN1-GFP labeling at two centrosomes shows metaphase chromosome. Insets (lower left, upper right), enlargement and semi-quantitative integrated intensity profile of centrioles. Bar, 5 μm. (**b**) The brighter CETN1-GFP signal represents the older centrosome as it co-stains more intensely for hCenexin1 and remains more intense throughout cell division (supplementary information, Fig. S1a). Bar, 5 μm. Lower left, merge. (**c, d**) Time-lapse images show that the mitotic MB is preferentially inherited by the daughter cell with the older centrosome in HeLa cells (c) and hESCs (d). Cells were imaged at the indicated times (hr:min) from telophase by phase-contrast microscopy (c) and from metaphase by DIC microscopy (d). Middle panel of (c) and left panel of (d), CETN1-GFP at centrosomes; enlargements and integrated intensity profiles show the daughter cell having the older

centrosome (c, upper; d, lower) inherits the MB^d (Time-lapse images: 9:59 in c; lower right image in d). Mitotic MB and MB^ds (c, d; arrows). MKLP1, MB^d marker (red); α-tubulin, mitotic MB and cell boundary marker (green); DAPI, DNA (blue). Bars, 10 μm (c, d).

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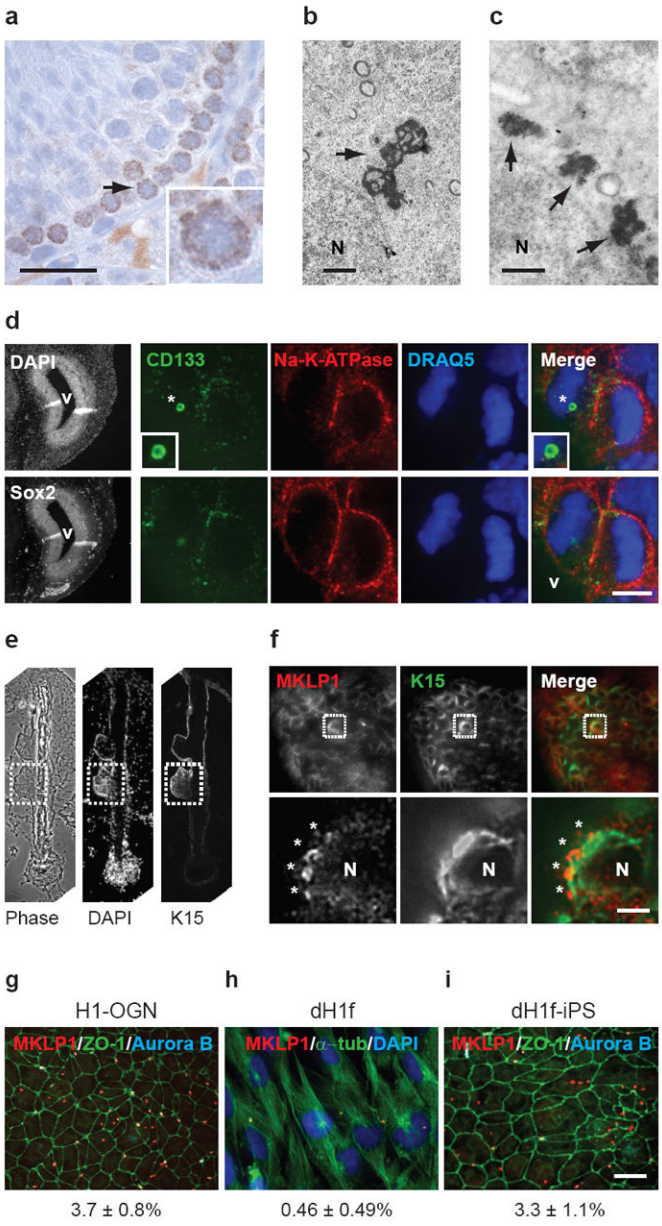

Figure 3.

MB[d]s accumulate in stem cells *in vivo* and *in vitro*. (**a**) Histological section through mouse seminiferous tubules labeled for MKLP1 shows several MKLP1+ puncta in cells of the basal layer where stem cells reside. Bar, 20 μm. Inset, enlargement of the cell (arrow) (**b, c**) Electron micrographs of mitotic MB (b, arrow) and multiple MB-like structures in interphase cells with similar shape and size in a juxtanuclear position (c, arrows) in basal cells of mouse seminiferous tubules. N, nucleus. Bars, 1 μm. (**d**) Representative planes of a neural progenitor cell in the ventricular zone (Sox2+, left-bottom panel) of an E13.5 mouse brain show that an intracellular MB[d] (asterisk) is associated with the ventricle-facing daughter in the asymmetrically dividing cell (top row). The bottom row emphasizes the position of paired chromosomes in a dividing anaphase cell. CD133, MB/MB[d] marker

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(green); Na-K-ATPase, cell-border marker (red); DRAQ5, DNA (blue); DAPI, DNA. Ventricle (V). Bar, 5 μm. Note that abscission occurs apically in these cells. (**e**) A histological section through a hair follicle (left, phase-contrast microscopy) stained for the stem cell marker keratin 15 to identify the bulge region (dotted box), the stem cell niche. DNA stain (DAPI) and the phase-contrast image show full follicle architecture. (**f**) Upper panels show MB^d-accumulating cells in the bulge region (boxed) colabeled with K15 and MKLP1. Enlargements (lower panels) of the boxed region highlight a cell with four MB^ds (asterisks). N, nucleus. Bar, 5 μm. (**g-i**) Quantitative analysis and representative images show a decrease in MB^d-accumulating cells upon the differentiation of pluripotent stem cells (g, H1-OGN) to fibroblast-like cells (h, dH1f), and an increase in MB^d-accumulating cells after reprogramming differentiated cells (h) to induced pluripotent stem cells (i, dH1f-iPS). (g-i) numbers refer to mean ± s.d., n=3. MKLP1, MB^ds; ZO-1, tight junctions; α-tubulin, microtubules; Aurora B, MBs. Bar, 10 μm.

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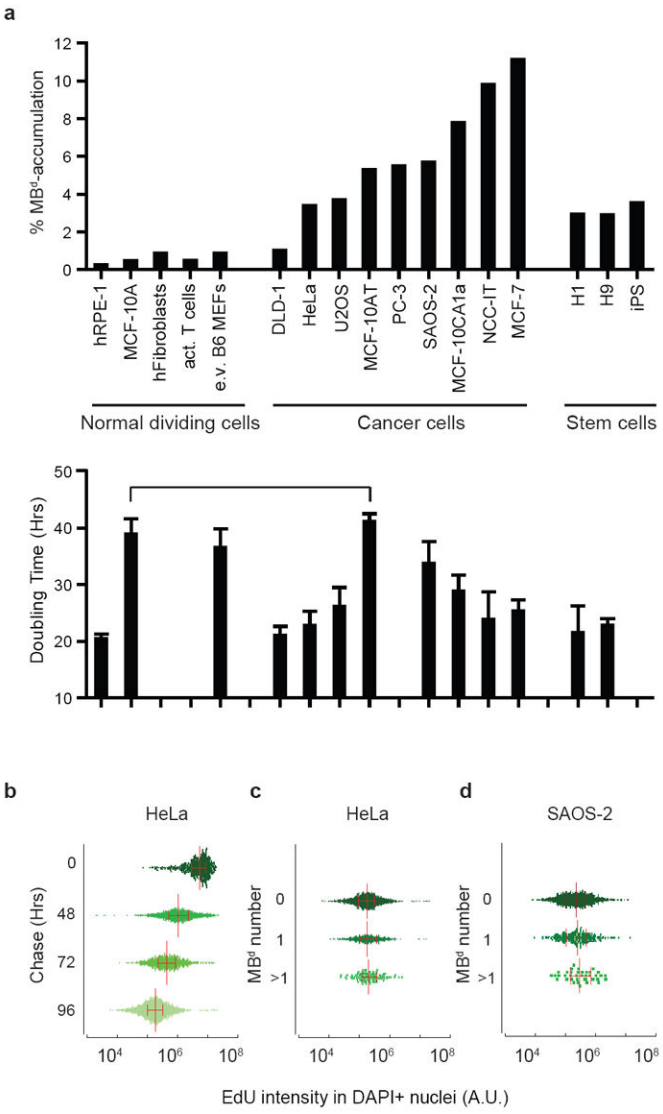

Figure 4.

MB^d-accumulation is high in stem cells and subpopulations of cancer cells and does not correlate with cell doubling time. (**a**) Percent of cells that accumulate MB^{d}s (>1) in a range of different cell types, as indicated. Below, doubling-times of representative cell lines aligned with MB^d-accumulation data. Data are presented as mean ± s.d.; Cell lines are examined in triplicate (MCF-10A, DLD-1, MCF-10AT, MCF-7, H1, and H9), or quadruplicate (e.v. B6 MEFs, HeLa, SAOS-2, and MCF-10CA1a), except hRPE-1 (n=6), U2OS (n=7) and NCC-IT (n=8). Horizontal line, cell lines with different MB^d-accumulation potential (14-fold) but similar doubling time. (**b**) Cells pulse-chased with EdU show a decrease in EdU intensity (x-axis) over time (y-axis), reflecting dilution of dye after cell divisions. (**c, d**) After a 96-hr chase period, EdU levels were compared between cells with MB^d numbers of >1, 1, and 0 (y-axis) in HeLa (c) and SAOS-2 cells (d). In both cases, no significant differences were noted (c, p=0.2101; d, p=0.5609, one-way ANOVA, with at least 800 cells analyzed for each experiment, n=3), indicating similar cycling rates among

different subpopulations of cells. (b-d) Each graph is a representative experiment. Cells analyzed shown by green points, median depicted by vertical red lines, and horizontal red lines with ticks illustrate the interquartile range.

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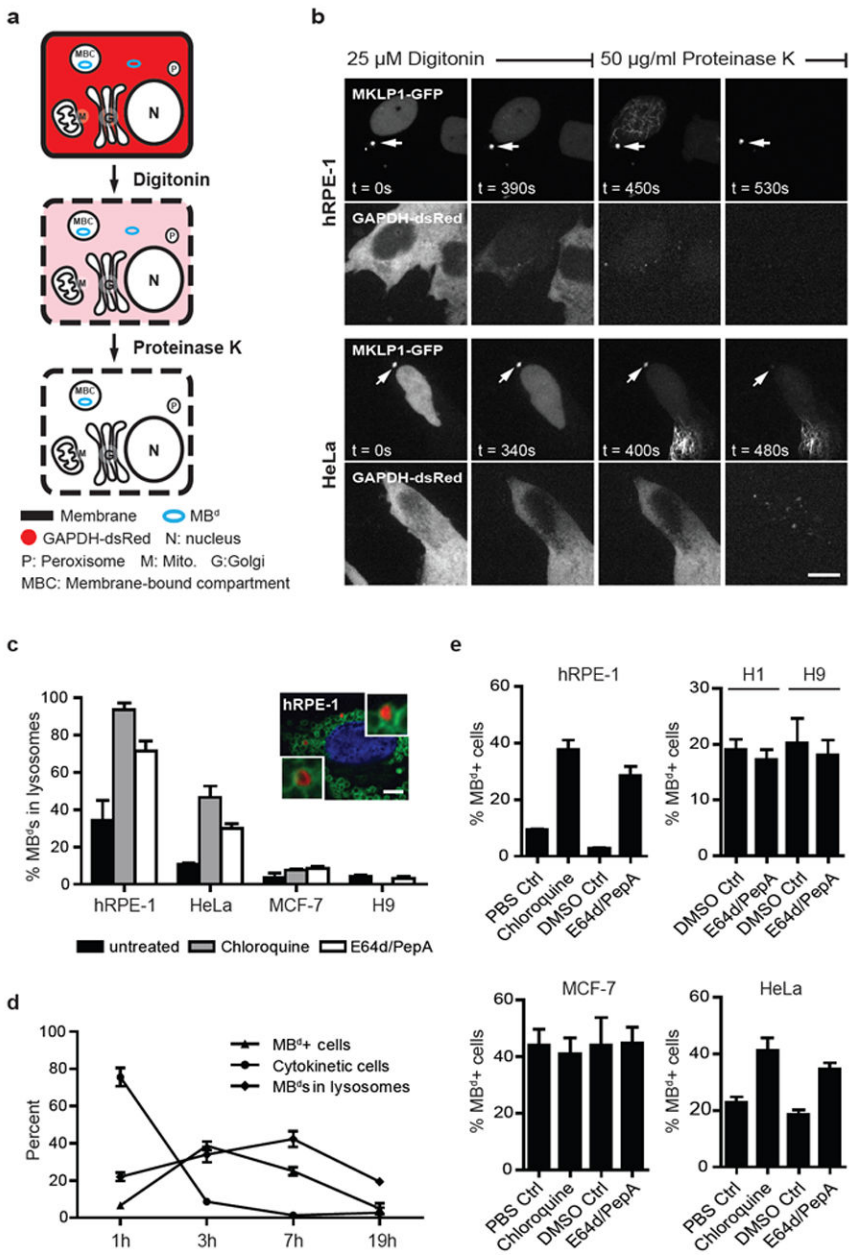

Figure 5.

MB^{d}s in stem and cancer cells evade membrane encapsulation and lysosomal degradation. (**a**) Depiction of fluorescence protease protection (FPP) assay. Digitonin selectively permeabilizes the plasma membrane but not internal membranes. Proteinase K degrades cytoplasmic components but membranous compartments remain intact. Under these conditions, MKLP1-GFP-labeled MB^{d}s (blue circle) in the cytoplasm will be degraded whereas those inside membrane-bound compartments (MBCs) will not. (**b**) MB^{d}s in MB^{d}-poor hRPE-1 cells are largely protected (~90% in membranous compartments, cells analyzed=10), whereas most MB^{d}s in HeLa cells are not (~27%, cells analyzed: 11), and are thus degraded in cytoplasm. Bar, 5 μm. (**c**) Graph depicting the presence of MB^{d}s in

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lysosomes upon chloroquine or E64d/pepstatin A (E64d/PepA) inhibition in hRPE-1 and HeLa cells, but not in MCF-7 and H9 hESCs. Chloroquine treatment of H9 hESCs is not included as it caused differentiation and cell death. A representative image of hRPE-1 cells inhibited by chloroquine is shown depicting two MB^ds inside lysosomes. MKLP1 and LAMP2 are used as MB^d (red) and lysosome (green) markers, respectively. DAPI, DNA (blue). n=100 MB^ds/treatment in each of the biological triplicates. Bar, 5 μm. (**d**) Graph showing the percent of MB^d+ cells (MB^d levels), the percent of MB^ds within lysosomes, and the percent of cells exiting cytokinesis following synchronization. MKLP1 and LAMP2 are used as markers as in (c). Note that MB^ds are transferred into only one of the two nascent daughter cells after abscission (Fig. 2d), so a 50% maximum will be expected for MB^d+ cells. The peak of MB^ds transferred to cells is 3 hours after plating followed by a peak of MB^ds entering lysosomes at 7 hours. (**e**) Both chloroquine and E64d/PepA treatments increase the percent of MB^d+ cells in hRPE-1 cells and HeLa cells (chloroquine: p=0.0021 and p=0.0187, respectively; E64d/PepA: p=0.0022 and p=0.0043, respectively; n=3 for all experiments). In contrast, lysosomal inhibition has no detectable effect on hESCs (H1, H9) and MCF-7 cancer cells. Data are presented as mean ± s.d. (c-e), except mean ± s.e.m. in hESCs (e).

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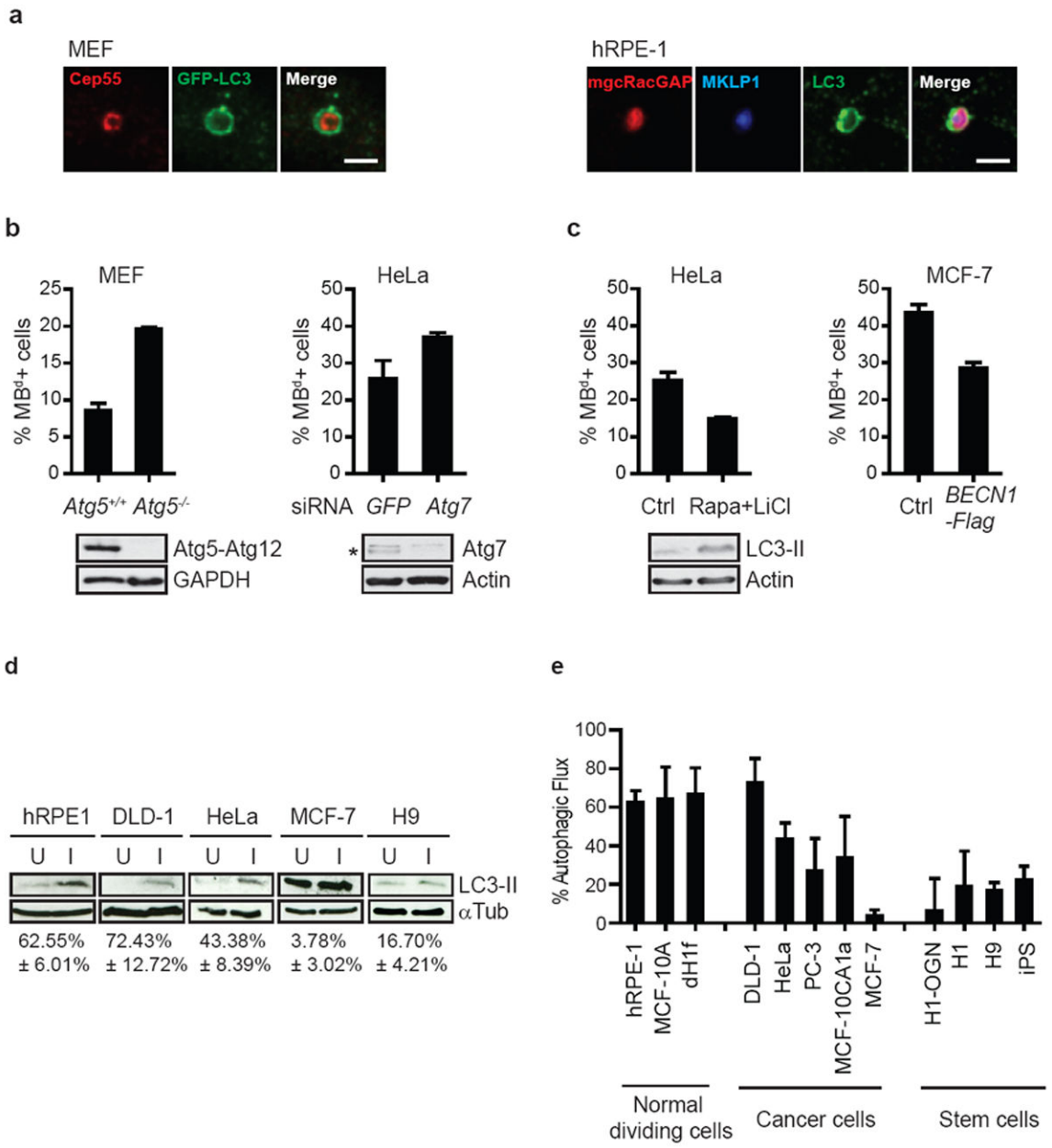

Figure 6.

Autophagy controls intracellular MB^d levels. (**a**) Single-plane confocal images of MB^{d}s within LC3-positive autophagosomes in MEFs expressing GFP-LC3 (left) and in hRPE-1 cells stained for endogenous LC3 (right). MB^d markers: Cep55, MKLP1, or mgcRACGAP. Autophagosomes: GFP-LC3 or LC3. Note that MKLP1 (blue) and mgcRACGAP (red) are co-localized (magenta) in the autophagosome (green), suggesting that MB^{d}s are sorted into autophagosomes. Bars, 2 μm. (**b**) Decreasing autophagy levels by deletion of *Atg5* gene (left, MEFs) or depletion of Atg7 by siRNA (right, HeLa) significantly increases the percent of MB^d+ cells (p=0.0019 and p=0.021, respectively, n=3). Immunoblots confirm loss of the Atg5-Atg12 conjugation in mutant cells and depletion of Atg7 (asterisk). (**c**) Rapamycin (Rapa) and lithium chloride (LiCl) co-treatment induces autophagy and decreases the

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percent of MB^d+ cells (left, HeLa; p=0.0056, n=3). Immunoblots showing increased LC3-II levels confirm autophagy induction. Induction of autophagy by over-expression of Flag-tagged BECN1 reduces the percent of MB^d+ cells (right, MCF-7; p=0.0008, n=4) (**d**) Representative immunoblots showing high autophagy levels in normal cells and low levels in stem cells and cancer cells. Autophagic flux (autophagic activity) was measured by changes in the levels of LC3-II, in the presence or absence of lysosomal inhibitors E64d/PepA. U, uninhibited. I, inhibited. Below, the average of the percent change in LC3-II levels after lysosomal inhibition from 3 experiments. α-tubulin, loading control. (**e**) Quantification of autophagic flux from 3 experiments in different cell lines. Normal dividing cells (MB^d-poor) typically have high autophagic flux, whereas stem and cancer cells (MB^d-rich) have low autophagic flux. The data are presented as mean ± s.d. (b-e).

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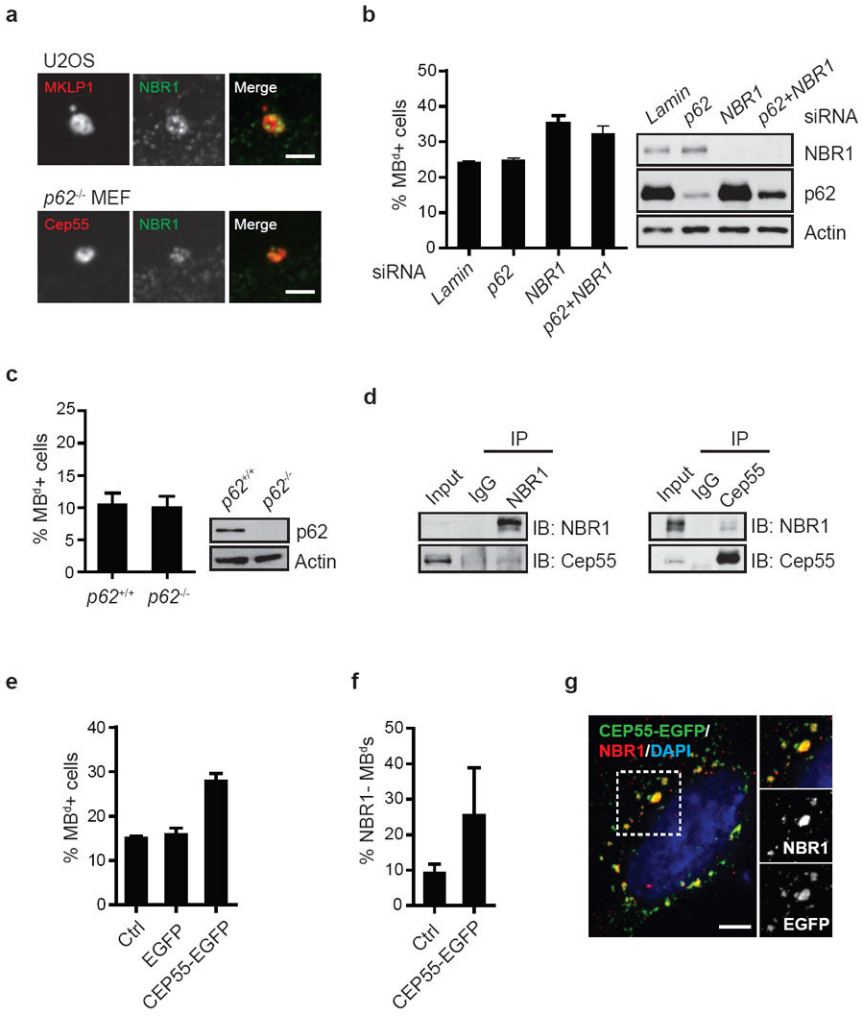

Figure 7.

NBR1 is a receptor for targeting MB^{d}s to the autophagy pathway. (**a**) Single-plane confocal images showing co-localization of the MB^{d} and the autophagic receptor, NBR1, in U2OS cells and *p62*-deleted MEFs. MB^{d} markers: MKLP1 or Cep55. Bar, 2 μm. (**b**) The percent of MB^{d}+ cells is significantly increased following the depletion of NBR1 (p=0.022, n=3), but not another autophagic receptor, p62. Co-depletion of NBR1 and p62 does not further increase MB^{d} levels over NBR1 depletion alone. (**c**) Deletion of the *p62* gene does not affect the percent of MB^{d}+ cells. For (b) and (c), immunoblots verify protein loss. (**d**) Co-immunoprecipitation reveals Cep55 and NBR1 form a complex. Precipitated proteins and 5% of the input material (Input) were analyzed by immunoblotting with antibodies against NBR1 or Cep55. (**e-g**) Over-expression of CEP55-EGFP increases the percent of MB^{d}+ cells (e; p=0.0007, n=3) and the percent of NBR1-negative MB^{d}s (f; p=0.0568, n=3), presumably by sequestering NBR1 (red) away from MB^{d}s in cells expressing CEP55-EGFP (green) as shown in (g), and consequently preventing MB^{d} degradation. The dotted box in (g) is enlarged (top right panel), and the labeling of NBR1 and CEP55-EGFP (middle and bottom right panel) are also presented. DAPI, DNA (blue). Bar, 5 μm. The data are presented as mean ± s.d. (b, c, e, and f).

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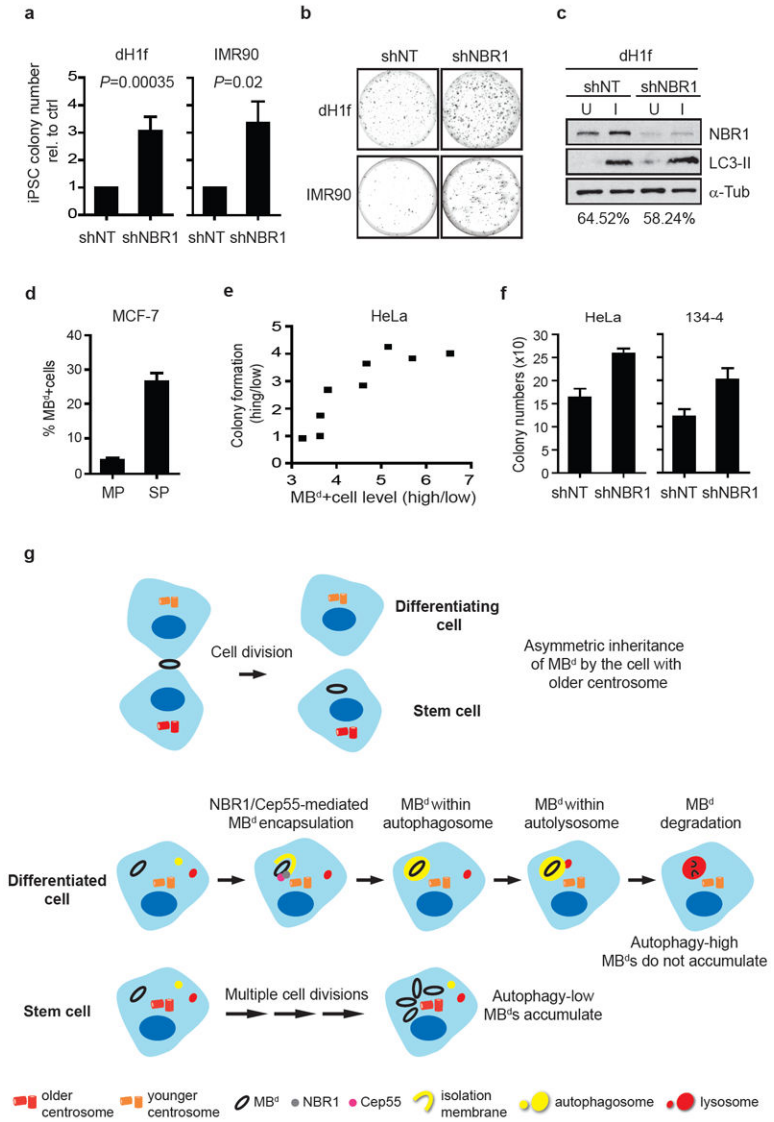

Figure 8.

MB^{d} enrichment increases reprogramming efficiency and enhances *in vitro* tumorigenicity. (**a-c**) Reprogramming is more efficient after MB^{d} enrichment. Differentiated cells (dH1f) and embryonic fibroblasts (IMR90) are reprogrammed after stable expression of either NBR1-specific shRNA (shNBR1) or non-targeting shRNA (shNT). Emerging iPSC colonies are scored based on Tra-1-60 expression[37]. (a, b) Cells depleted of NBR1 to increase MB^{d} levels show an increase in iPSC colony formation (a, dH1f: 3.1±0.5-fold, n=15, p=0.00035; IMR90: 3.4±0.8-fold, n=3, p=0.02; data are mean ± s.e.m.) but insignificant changes in autophagic activity (c) over shNT control. (b) Representative plates with Tra-1-60-immunostained iPSC colonies. Immunoblot (c, top) and densitometry (c, bottom; percent of autophagic flux) show representative result (n=3); α-tubulin, loading control. (**d**) MCF-7 side-population (SP) cells have a significantly higher percentage of MB^{d}+ cells over the non-SP population (MP; p=0.0015, n=3; data are mean ± s.d.). (**e**, **f**) MB^{d} enrichment in cancer cells leads to increased anchorage-independent growth. MKLP1-GFP-expressing

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HeLa cells are separated into "MB^d high" and "MB^d low" subpopulations. An increase in the "MB^d high" over "MB^d low" ratio is associated with an increase in soft-agar colony formation (e). No significant difference was observed when the enrichment of MB^d high subpopulation was less than 3-fold. More soft-agar colonies are formed when MB^ds are enriched by NBR1-depletion (shNBR1) in HeLa (f, left; p=0.0012, n=3) and mouse 134-4 cells (f, right; p=0.0086, n=3); control, shNT. Data are mean ± s.d., and the colony number (e, f) is the sum of INT-violet-stained colonies from 10 random fields. (**g**) Model for MB^d fate in cells. The newly-formed MB^d is preferentially inherited by the daughter cell with the older centrosome (top panel). The inherited MB^d (black ring) is recognized by binding of the NBR1 autophagic receptor (grey circle) with the MB protein Cep55 (magenta). The MB^d is then encapsulated by the autophagosome (yellow circle), and degraded after fusion of autophagosome and lysosome (red circle) in differentiated cells. This pathway prevents MB^d-accumulation. In contrast, stem cells efficiently accumulate MB^ds through successive divisions and evasion of NBR1-mediated autophagy. Additionally, differentiated and stem cells possess overall high and low autophagic activity, respectively.